USE OF A FORMULATION FOR CONTROLLING EXPRESSION OF A TARGET GENE

The present invention relates to the use of a formulation as a control mechanism for the expression of a target gene in an organism such as a plant, as well as to certain formulations useful in this way.

Gene expression is controlled by regions upstream (5') of the protein encoding region, commonly referred to as the "promoter". A promoter may be constitutive, tissue-specific, developmentally-programmed or inducible.

Manipulation of crop plants to improve characteristics (such as productivity or quality) requires the expression of foreign or endogenous genes in plant tissues. Such genetic manipulation therefore relies on the availability of means to control gene expression as required; for example, on the availability and use of suitable promoters which are effective in plants. It is advantageous to have the choice of a variety of different promoters so that the most suitable promoter may be selected for a particular gene, construct, cell, tissue, plant or environment. A range of promoters are known to be operative in plants.

The term "inducible promoter" includes promoters which may be induced chemically. Particularly useful promoters are promoter sequences which are controlled by the application of an external chemical stimulus. The external chemical stimulus may be an agriculturally acceptable chemical, the use of which is compatible with agricultural practice and is not detrimental to plants or mammals. This allows particular gene expression to be controlled at particular stages of plant growth or development, by the presence or absence of a chemical which can be applied to the plants or seeds, for example by spraying or using known seed coating techniques. These are also known as a gene "switch" promoters.

The gene which is under the control of the inducible promoter may be the gene which gives rise to the desired characteristic or phenotype itself, or the inducible promoter may control expression of a repressor protein which inhibits expression of a target gene, for example by interacting with an operator sequence upstream of the target gene so as to prevent expression of the gene (for example as known in the bacterial *tet* and *lac* operator/repressor systems). In a further alternative, the gene under the control of the inducible promoter may express a protein which interacts with another protein to inhibit the

activity thereof, as for example in the barnase/barstar system which barnase will inhibit or kill cells in the absence of barstar.

Gene switches of this type are known in a wide variety of applications. These include the production of reversible male sterility, a feature which is highly desirable in hybrid plant production as described for instance in WO 90/08830. Other applications of such promoters include in germplasm protection, where containment of particular crop plants, in particular transgenic plants, and the control of volunteers is necessary and also in the prevention of pre-harvesting sprouting as described in WO 94/03619.

Many organisms have mechanisms which allow them to metabolise chemicals such as alcohols or ketones, for example by the production of alcohol dehydrogenase enzymes. The promoters of these systems may be useful in gene switches as the promoters may be inducible by the presence of the target alcohol or ketone.

One such example can be found in the fungal organism *A nidulans* which expresses the enzyme alcohol dehydrogenase I (ADH1) encoded by the gene *alcA* only when it is grown in the presence of various alcohols and ketones. The induction is relayed through a regulator protein encoded by the *alcR* gene which is constitutively expressed. In the presence of inducer (alcohol or ketone), the regulator protein activates the expression of the *alcA* gene. The regulator protein also stimulates expression of itself in the presence of inducer. This means that high levels of the ADH1 enzyme are produced under inducing conditions (ie when alcohol or ketone are present). Conversely, the *alcA* gene and its product, ADH1, are not expressed in the absence of inducer. Expression of *alcA* and production of the enzyme is also repressed in the presence of glucose.

Thus, the *alcA* gene promoter is an inducible promoter, activated by the *alcR* regulator protein in the presence of inducer (ie by the protein/alcohol or protein/ketone combination). The *alcR* and *alcA* genes (including the respective promoters) have been cloned and sequenced (Lockington RA *et al*, 1985, Gene, 33:137-149; Felenbok B *et al*, 1988, Gene, 73:385-396; Gwynne *et al*, 1987, Gene, 51:205-216).

Alcohol dehydrogenase (*adh*) genes have been investigated in certain plant species. In maize and other cereals they are switched on by anaerobic conditions. The promoter region of *adh* genes from maize contains a 300 bp regulatory element necessary for expression under anaerobic conditions. However, no equivalent to the *alcR* regulator protein

has been found in any plant. Hence the *alcR/alcA* type of gene regulator system is not known in plants. Constitutive expression of *alcR* in plant cells does not result in the activation of endogenous *adh* activity.

WO 93/21334 describes the production of transgenic plants which include such a system as a gene switch. This document specifically describes a chemically inducible plant gene expression cassette comprising a first promoter operatively linked to a regulator sequence which encodes a regulator protein, and an inducible promoter operatively linked to a target gene, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene. In particular, the *alcR/alcA* system is utilised in the constructs. Exogenous chemical inducers which are applied in this case include those described by Creaser *et al.*, J. Biochem. (1984) 225, 449-454) such as butan-2-one (ethyl methyl ketone), cyclohexanone, acteone, butan-2-ol, 3-oxobutyric acid, propan-2-ol and ethanol.

For agricultural purposes, the application of simple alcohols may be preferred as such chemicals are relatively cheap and are likely to be readily degraded. They therefore present less of an environmental burden. However, such chemicals are often highly volatile and therefore difficult to handle in an agricultural context, as large volumes of chemical may be lost during spraying. One of the problems associated with the use of the known inducers is, therefore, their ability to evaporate rapidly before they can be effectively taken up by a plant.

It is therefore desirable to use a chemical inducer which may be taken up by a plant more effectively than the known inducers thereby allowing better control of expression of a target gene.

The present invention therefore seeks to overcome the problems associated with the prior art, by formulating the chemical inducer.

According to a first aspect of the present invention there is provided the use of a formulation comprising the components:

(a) a volatile chemical inducer;

(b) a polyethoxylated C_{10}-C_{20} alcohol or a trisiloxane polyethoxylate and

(c) a diluent;

for controlling expression of a target gene in an organism having a chemically-inducible gene expression cassette comprising an inducible promoter operatively linked to the target gene wherein the inducible promoter is induced by the application to the organism of (a) above.

Suitably, the organism is a plant such as a crop plant.

The diluent (c) may be, for example, water.

According to a second aspect of the present invention, there is provided a method of controlling expression of a target gene in an organism, such as a plant, comprising transforming the organism with a chemically-inducible plant gene expression cassette comprising an inducible promoter operatively linked to the target gene wherein the inducible promoter is induced by the application to the organism of a formulation as described above.

Suitably the expression system utilises a regulator protein.

Thus according to a third aspect of the present invention there is provided a method of controlling expression of a target gene in a plant comprising transforming the plant with a chemically-inducible plant gene expression cassette comprising a first promoter operatively linked to a regulator sequence which encodes a regulator protein, and an inducible promoter operatively linked to the target gene, the inducible promoter being activated by the regulator protein in the presence of a formulation as defined above, the method comprising applying to the plant a formulation as defined above, whereby application of the inducing formulation causes expression of the target gene.

The nature of component (a) in the above formulations depends upon the character of the inducible promoter present in the expression system. However, a particular examples of component (a) are a C_1-C_6 alcohol or a C_3-C_9 ketone, and preferably, ethanol or propan-2-ol. These components act as inducers for example, of the *alc* switch system as described in detail below.

Component (b) of the formulation used as described above, preferably, a polyethoxylated oleyl, lauryl, stearyl or cetyl alcohol. It is more preferably a polyoxyethylene-oleyl alcohol having a mean molar ethylene oxide content in the range of 0 to 35 and more preferably in the range of 2 to 20. It is most preferably a polyoxyethylene-(2)-oleyl alcohol, a polyoxyethylene-(10)-oleyl alcohol or a polyoxyethylene-(20)-oleyl alcohol. Component (b) is, however, preferably a polyoxyethylene-(20)-oleyl alcohol (the

number in brackets indicates the mean ethylene oxide content per molecule). Such products are commercially available as BRIJ 92™, BRIJ 97™ and BRIJ 98™.

Preferably, component (b) of the formulation is at a concentration of about 0.5% wt/wt or less. It is preferably at a concentration between about 0.2% wt/wt and 0.5% wt/wt.

In an alternative embodiment, the formulation used in the methods of the invention includes as component (b), a hydrogen or methyl end-capped trisiloxane polyethoxylate In particular, component (b) is a methyl end-capped trisiloxane polyethoxylate. The methyl end-capped trisiloxane polyethoxylate preferably has a mean molar ethylene oxide content of between 4 and 12 per molecule and is most preferably 8 per molecule. Such products are commercially available as SILWET 77™ (SILWET is a trademark of Witco).

Preferably, the methyl end-capped trisiloxane polyethoxylate is at a concentration of about 0.5% wt/wt or less. It is preferably at a concentration between about 0.2% and 0.5% wt/wt.

Component (a) of the formulation used in the above-described methods is preferably at a concentration of about 5% wt/wt or less. It is preferably at a concentration between about 2% and 5% wt/wt.

The inducible promoter according to a preferred embodiment of the third aspect of the present invention is the *alcA* inducible promoter sequence and the regulator sequence encodes the *alcR* regulator protein.

Component (c) of the formulation is preferably at a concentration between about 90% and 98% wt/wt.

Certain formulations used in accordance with the above-described methods are novel and provide a further aspect of the invention.

Thus, according to a fourth aspect of the present invention there is provided an agricultural formulation comprising the components:

(a) a volatile chemical inducer of an inducible promoter;

(b) a trisiloxane polyethoxylate; and

(c) a diluent.

As mentioned above, component (b) is suitably a hydrogen or a methyl end-capped trisiloxane polyethoxylate. and preferaably is a methyl end-capped trisiloxane polyethoxylate. Suitably, the methyl end-capped trisiloxane polyethoxylate has a mean

molar ethylene oxide content of between 4 and 12 per molecule, for example 8 per molecule. Suitably, component (a) is at a concentration between about 2% and 5% wt/wt.

According to a fifth aspect of the present invention, there is provided an agricultural formulation, comprising

(a) a C_1-C_6 alcohol inducer of an inducible promoter in an amount of less than 5%wt/wt;

(b) a polyethoxylated C_{10}-C_{20} alcohol; and

(c) water.

In this case, component (b) of the formulation is suitably a polyethoxylated oleyl, lauryl, stearyl or cetyl alcohol. and preferably a polyoxyethylene-oleyl alcohol. Suitably, the polyoxyethylene-oleyl alcohol has a mean molar ethylene oxide content in the range of 2 to 20, such as a polyoxyethylene-(2)-oleyl alcohol, a polyoxyethylene-(10)-oleyl alcohol or a polyoxyethylene-(20)-oleyl alcohol. Again component (a), which is preferably ethanol or propan-2-ol, is suitably at a concentration between about 2% to less than 5% wt/wt. The concentration of component (B) is preferably about 0.5% wt/wt or less.

According to a sixth aspect of the present invention, there is provided an agricultural formulation comprising

(a) a C_3-C_9 ketone which is able to act as a chemical inducer of an inducible promoter;

(b) a polyethoxylated C_{10}-C_{20} alcohol; and

(c) a diluent.

Preferably, in these formulations, component (a) is at a concentration between about 2% and 5% wt/wt. As before, component (b) may be a polyethoxylated oleyl, lauryl, stearyl or cetyl alcohol, and is preferably a polyoxyethylene-oleyl alcohol, with a mean molar ethylene oxide content in the range of 2 to 20. Particular examples of component (b) are polyoxyethylene-(2)-oleyl alcohol, polyoxyethylene-(10)-oleyl alcohol and polyoxyethylene-(20)-oleyl alcohol.

A preferred embodiment of the present invention is a formulation comprising the components: a. ethanol; b. polyoxyethylene-(20)-oleyl alcohol and c. a diluent wherein

component a. is at a concentration of 2% wt/wt and component b. is at a concentration of 0.5% wt/wt.

An even more preferred embodiment of the present invention is a formulation comprising the components: a: ethanol; b. methyl end-capped trisiloxane polyethoxylate having a mean molar ethylene oxide content of 8 and c. a diluent wherein component a. is at a concentration of 2% wt/wt and component b. is in the concentration of 0.5% wt/wt.

An advantage of the present invention is that the formulation is taken up by the organism such as the plant more effectively than when known inducers alone are used. The formulation according to the present invention therefore increases the effectiveness of ethanol as an inducer of the *alcA/alcR* promoter.

It has been surprisingly found that the use of polyoxyethylene-oleyl alcohol surfactants and/or methyl end-capped trisiloxane polyethoxylate adjuvants in combination with the known chemical inducers for the *alcA/alcR* promoter increases uptake of the formulation by a plant significantly, thereby allowing greater control of a target gene which may be operatively linked to the inducible promoter.

Other additives, such as antibacterial compounds, dispersants, wetter compounds and anti-evaporants may be added.

The invention is particularly applicable in the context of a plant gene expression system comprising

(i) a first promoter operatively linked to a regulator sequence which encodes a regulator protein; and

(ii) an inducible promoter operatively linked to a target gene, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer, which is the formulation defined above, whereby application of the inducer causes expression of the target gene.

Such gene expression systems may be contained within a plant cell as well as plant tissue or a plant comprising plant cells as defined above and plants, or seeds, derived therefrom.

The term "volatile chemical inducer" means any chemical which is capable of inducing a chemically inducible promoter system and which may evaporate rapidly before it can be effectively taken up by a plant.

The term "C_1-C_6 alcohol" includes methanol, ethanol, *n*-propanol, *iso*propanol, *n*-butanol, *iso*-butanol, *sec*- butanol, *tert*-butanol, *n*-pentanol, *n*-hexanol and cyclohexanol and unsaturated analogues thereof.

The term "C_3-C_9 ketone" includes acetone, butanone, pentanone, hexanone, cyclohexanone, heptanone, octanone, nonanone and aromatic ketones such as acetophenone.

The term "target gene" with reference to the present invention means any gene of interest. It may comprise any gene which is required to be introduced into a plant in order to modify the characteristics thereof. The target gene may be an endogenous plant gene or a foreign gene, and may be a single gene or a series of genes. The target gene sequence may encode at least part of a functional protein or an antisense sequence. An inducible promoter, therefore, when linked to an endogenous or foreign gene and introduced into a eukaryote by transformation, provides a means for the external regulation of expression of that gene.

The term "expression cassette", which is synonymous with terms such as "construct", "hybrid" and "conjugate" - includes a gene of interest directly or indirectly attached to an inducible promoter. An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence intermediate the promoter and the target gene. The same is true for the term "fused" in relation to the present invention which includes direct or indirect attachment. Such constructs also include plasmids and phage which are suitable for transforming a cell of interest. The expression cassette of the present invention may also comprise additional components such as a regulator sequence operatively linked to a further promoter, the inducible promoter being activated by the regulator protein in the presence of an exogenous inducer.

The term "expression system" means that the system defined above can be expressed in an appropriate organism, tissue, cell or medium. In this regard, the expression system of the present invention may comprise one or more expression cassettes and may also comprise additional components that ensure the increased expression of the target gene by use of the inducible promoter.

Any transformation method suitable for the target plant or plant cells may be employed, including infection by *Agrobacterium tumefaciens* containing recombinant Ti plasmids, electroporation, microinjection of cells and protoplasts, microprojectile bombardment, bacterial bombardment, particularly the "fibre" or "whisker" method, and

pollen tube transformation. The transformed cells may then in suitable cases be regenerated into whole plants in which the new nuclear material is stably incorporated into the genome. Both transformed monocot and dicot plants may be obtained in this way. Reference may be made to the literature for full details of the known methods.

Examples of genetically modified plants which may be produced include dicotyledonous and monocotyledonous plants such as field crops, cereals, fruits and vegetables such as: canola, sunflower, tobacco, sugarbeet, cotton, soya, maize, wheat, barley, rice, sorghum, tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, potatoes, carrot, lettuce, cabbage, onion.

Various preferred features and embodiments of the present invention will now be described by way of non-limiting example and with reference to the accompanying drawings in which:-

Figure 1 shows CAT induction by foliar spray using ethanol alone (2% wt/wt) and ethanol (2% wt/wt) in combination with BRIJ 92, 97 and 98™ (0.5% wt/wt) formulations (5 repeats/ treatment);

Figure 2 shows the relationship of increasing SILWET L-77™ adjuvant concentration (0.2% wt/wt and 0.5% wt/wt) with 2% wt/wt ethanol and ethanol (2% wt/wt) alone to CAT activity in a glasshouse leaf spray test;

Figure 3 shows the induction of Alc-GUS oilseed rape by application of 5% wt/wt ethanol and 0.5% wt/wt formulation to the upper leaf surface of oilseed rape leaves ('T' = target leaf, 'A' = adjacent leaf from the same plant); and

Figure 4 shows the structure of Silwett L77™.

The Table below gives details of the components of each surfactant/ adjuvant described in the Examples.

Surfactant/ adjuvant	Components	Manufacturer
BRIJ 92™	Polyoxyethylene-(2)-oleyl alcohol	ICI Surfactants
BRIJ 97™	Polyoxyethylene-(10)-oleyl alcohol	ICI Surfactants
BRIJ 98™	Polyoxyethylene-(20)-oleyl alcohol	ICI Surfactants
SILWET L-77™	Methyl end-capped trilsiloxane polyethoxylate (8) -	

	84% Allyloxypolyethyleneglycol methyl ether - 16% (structure shown in Figure 4)	Witco

EXAMPLE 1

Component	Amount
a. Ethanol	1.99g
b. BRIJ 92™	0.51g
c. Water	97.86g

99.86g of a 20% wt/wt ethanol solution (20.01g ethanol in 980.56g water) were mixed in a vial with 0.51g Brij 92™. The sample was shaken before use.

EXAMPLE 2

Component	Amount
a. Ethanol	2.00g
b. BRIJ 97™	0.52g
c. Water	98.05g

100.05g of a 20% wt/wt ethanol solution (20.01g ethanol in 980.56g water) were mixed in a vial with 0.52g Brij 97™. The sample was shaken before use.

EXAMPLE 3

Component	Amount
a. Ethanol	1.99g
b. BRIJ 98™	0.50g
c. Water	97.9g

99.90g of a 20% wt/wt ethanol solution (20.01g ethanol in 980.56g water) were mixed in a vial with 0.50g Brij 98™. The sample was shaken before use.

EXAMPLE 4

Component	Amount
a. Ethanol	2.02g

b. SILWET L-77™ 0.50g

c. Water 99.27g

101.30g of a 20% wt/wt ethanol solution (20.01g ethanol in 980.56g water) were mixed in a vial with 0.50g Silwet L77™. The sample was shaken before use.

EXAMPLE 5

In order to see the effect of the above formulations (see Figures 1 and 2) on 8 week old Alc-CAT ('Alc' refers to the CaMV35S:AlcR, AlcA/CaMV35S:CAT system) tobacco plants, the plants were hand-sprayed to run-off on the adaxial leaf surface on the V5 leaf with an adjuvant-(or surfactant) ethanol mixture as described in Examples 1 to 4 above. The plants were left for 72 h under glasshouse growth conditions with root water irrigation and the target leaf sampled and assayed for CAT (chloramphenical acetyl transferase) activity using a CAT ELISA kit (Boehringer Mannheim). Plants sprayed with ethanol alone were used as controls. 5 repeats per treatment were used (error bars indicate the standard deviation).

It can be seen that the use of polyoxyethylene-(20)-oleyl alcohol (BRIJ 98™) as a surfactant allows greater expression of the CAT reporter gene than the use of ethanol alone. The use of a methyl end-capped trisiloxane ethoxylate with a mean molar ethylene oxide content of 8 per molecule (SILWET L-77™) in combination with ethanol is, however, able to increase expression of the CAT gene to an even greater extent than polyoxyethylene-(20)-oleyl alcohol (BRIJ 98™) in combination with ethanol. In particular, the use of BRIJ 98™ or SILWET L-77™ , each at 0.2% wt/wt and at 0.5% wt/wt, significantly increased reporter gene expression and uptake in AlcCAT tobacco plants when applied as a mixture with ^{14}C-ethanol (at concentrations varying between about 1% to 5% wt/wt) to the upper surface of the leaves. Phosphoimage studies were used to detect ^{14}C-ethanol uptake.

It was found that the use of ethanol alone resulted in a 2.1% uptake by the tobacco plants. The addition of increasing concentrations of SILWET L-77™ from 0.2% to 0.5% wt/wt resulted in a 61% to 78% increase in CAT expression compared to ethanol alone at a concentration of 5% wt/wt. In ^{14}C-ethanol formulation studies with BRIJ98™, a 68% increase in ethanol uptake was seen compared to ethanol alone.

Significant reporter gene activation was also seen with these formulations in potato and oil seed rape.

EXAMPLE 6

Homozygous 8-week-old soil-grown transgenic Alc-GUS (CaMV 35S:AlcR, AlcA/CaMV 35S:CAT construct) oilseed rape plants were hand-sprayed to run-off on the adaxial leaf surface on the 5 th leaf from the apex with an adjuvant-ethanol mixture (5 % wt/wt ethanol and 0.5 % wt/wt adjuvant). The plants were left for 72 h under glasshouse growth conditions with root water irrigation. Target leaves were sampled and assayed for GUS (β-Glucuronidase) activity and values normalised against protein concentration. Plants sprayed with ethanol without formulation were used as controls ('Ethanol alone'). Five replicate plants were used per treatment. As can be seen from Figure 3, error bars indicate standard deviation, 'CaMV 35S/10' signifies a tenth of the GUS activity in a constitutive control plant containing the cauliflower mosaic virus driving the GUS reporter gene, 'Silwett' signifies Silwett L77™' and 'EtOH' signifies a 5 % wt/wt ethanol leaf-spray treatment without formulation.

Other modifications of the present invention will be apparent to those skilled in the art without departing from the scope of the present invention.